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# Biomass and lipid production of Chlorella protothecoides under heterotrophic cultivation on a mixed waste substrate of brewer fermentation and crude glycerol

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# BIOMASS AND LIPID PRODUCTION OF CHLORELLA PROTOTHECOIDES UNDER HETEROTROPHIC CULTIVATION ON BREWER FERMENTATION WASTE AND CRUDE GLYCEROL

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Accepted by:
Dr. Terry H. Walker, Committee Chair
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Dr. David Ladner

**ABSTRACT**

Biodiesel production using *Chlorella protothecoides* microalgae species has become an attractive topic due to the intense requirements of renewable energy in past decades. While, the expensive capital cost of the carbon and nitrogen substrates for algae growth is becoming a restrictive problem in this filed. In this study, heterotrophic cultivation of *Chlorella protothecoides* in the dark was conducted in 500mL shake flasks. *Chlorella protothecoides* growth in mixed substrate of brewer fermentation and biodiesel crude glycerol by-products containing a relatively high concentration of carbon and nitrogen was discussed and compared with that in microalgae basal medium which was supplied with 30 g/L pure glucose and 4 g/L yeast extract as its carbon and nitrogen sources. An old *Chlorella protothecoides* which was stored for six month and a new *C. protothecoides* strains were inoculated in three different batches supplying with two tested medium. The results of biomass accumulation (g/L), lipid concentration (g/L), total organic carbon (g/L), total nitrogen (g/L) and accumulated biomass and lipid productivities (g/L/day) were discussed and compared in this study.

For the heterotrophic experiments, the biomass concentration of the old and new *C. protothecoides* strains in basal medium with supplement of pure glucose and yeast extract (BM-GY) were 14.47 g/L and 11.43 g/L on the sixth day, respectively. Using mixed by-product substrate, the biomass concentration on day 6 reached 14.07 g/L in the old strains batch and 12.73 g/L in the new strains batch. There were no significant differences of lipid content between BM-GY

group and mixed waste group, while, the new strains achieved higher lipid contents than those of the old strains. Approximately 81.5 wt% of total organic carbon and 65.1 wt% of total nitrogen in the mixed waste were removed during the cultivation period. The accumulated lipid productivities achieved in BM-GY medium were 2.07 g/L/day of the old strains and 1.61 g/L/day of the new strains. In the mixed waste groups, lipid productivitiy of the old strains was 2.12 g/L/day and 1.81 g/L/day of the new strains. Based on the data analysis, optical density was a reasonable indicator to predict biomass accumulation in further studies and there was a linear relationship between the optical density and cell concentration.

In summary, this study showed the potential usage of industrial waste stream such as brewer fermentation waste and crude glycerol for *C. protothecoides* fermentation to produce biofuel. These alternative sources of microalgae substrates could lower the capital cost as well as meet the goal of environmental friendly technologies within the biofuel industry.

## DEDICATION

This thesis is dedicated to all the people who have supported me a lot in my life and on my study: my parents, Shouzhong Feng and Guoli Yan, my friends, Bin Zhang, Yang Zhou, Yifan Du, Karthik Gopalakrishnan, Muriel Steele and Arpan Jain for all their help. I also thank all my other friends in the United States and in China for their understandings and love.

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# CHAPTER I

# INTRODUCTION

## 1.1 Introduction

With increasing concerns on the continued use of fossil fuels, biofuels, which is a renewable and green alternative, now have received a large amount of attention all across the world. Production of biodiesel, however, may use oil crops or waste oil that may not match the existing demand for traditional fuels (Christenson and Sims 2011). Fuels derived from algae appears to be a more promising feedstock in recent years since algae are regarded as a favorable biofuel source due to its characteristics of potential high oil content and fast generation of biomass. Nutrients like nitrogen, phosphorus, and potassium are significant for algae plant growth and these kinds of nutrients may be derived from some waste stream such as brewer fermentation waste and crude glycerol from biodiesel production. Many studies focused on using different industrial waste for algae treatments (Mallick 2002; W.J.Oswald, et al. 1957; S.K.Mehta and J.P.Gaur 2005). Because brewer fermentation wastewater effluent from an anaerobic digester contains high concentrations of nitrogen, mostly in the form of ammonia nitrogen, and inorganic phosphorous due to the biotransformation of proteins and solids (Cui, Lee and Kim 2011), this kind of waste source may be a good cultivated nitrogen source for treating algae. Meanwhile, the biodiesel crude glycerol contains abundant organic carbon that can be utilized as a carbon

source. An assumption of using the mixture of these two waste streams to supply enough carbon, nitrogen and other essential elements for algae growth instead of pure sugars and organic nitrogen such as yeast extract is a potential way to decrease the capital and operating costs of biodiesel production.

### 1.2 Objectives

In this study, brewer fermentation waste has been used as a major part to supply nutrients that algae require and biodiesel by-product crude glycerol has been mixed with brewer fermentation waste to provide enough carbon source in the medium. Basic medium with supplement of glucose and yeast extract (BM-GY) was set as a control group. The influences of heterotrophic cultivation, different carbon and nitrogen substrates were analyzed under batch-mode fermentation process in shake-flasks. The main objectives of this research are:

1. To characterize algae growth conditions using shake-flasks under heterotrophic cultivation.
2. To study the characteristic of brewer fermentation waste and crude glycerol and determine their potentials as substrates for *Chlorella protothecoides*.
3. To study and evaluate the biomass and lipid accumulation using substrate of brewer fermentation waste and crude glycerol
4. To compare the results of *Chlorella protothecoides* growth rate and biomass and lipid yields for traditional basal medium and mixed waste substrates.

5. To compare the performance of biomass accumulation, lipid content, biomass and lipid productivities of an old and a new *Chlorella protothecoides.*

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# CHAPTER II

# LITERATURE REVIEW

## 2.1 Microalgae

### 2.1.1 Biological characteristics of microalgae

Microalgae, a group of unicellular microorganisms, comprise of a large group of photosynthetic and heterotrophic organisms, which have great potential for cultivation as energy crops. Algae species typically exist widely in freshwater and marine systems. As one of the most important species on earth, microalgae have the capability of photosynthesis, which use carbon dioxide to produce a large amount of the oxygen in the atmosphere.

The major cellular components contained of microalgae are 25-54 wt% lipid, 17-24 wt% carbohydrates, 11-26 wt% protein, 7-8% RNA, 3-4% DNA and 9-14% other elements (Bumbak, et al. 2011). Microalgae are divided into nine eukaryotic classes named Chlorophyta, Chlorarachniophyta, Cryptophyta, Dinophyta, Euglenophyta, Glaucophyta, Haptophyta, Heterokontophyta and Rhodophyta, and two prokaryotic classes that are Cyanophyta and Prochlorophyta (Mutanda, et al. 2011). In addition, this kind of microorganism may grow under all three major environmental conditions: autotrophic condition using inorganic carbon as C source, heterotrophic condition using organic carbon as C source and mixotrophic condition which can use both inorganic carbon and organic carbon as its carbon source.

### 2.1.2 Microalgae strains selection

To select proper algal strains for biofuel production, there are several important factors needed. Firstly, the lipid content is regarded as one of the most significant aspects. Generally, as reported in Table 2.1, microalgae contains oil levels between 20 and 75 % by weight of dry biomass and algae strains with a faster growth rate usually have a relatively lower oil content (Ghasemi, et al. 2012). Other considerations include: how the algae strains can resist environmental condition changes; the ability of competing with other microalgae species or bacteria; the types and amounts of available nutrient used by algae, whether the algae strains has the possibility of obtaining other valuable chemicals from surroundings; and metabolic methods of a specific microalgae that will be further discussed in this review.

The species used in this study is the green microalga *Chlorella protothecoides* that may be grown both autotrophically and heterotrophically. Further, *Chlorella* microalgae is one of the most understood species in research field compared with other various algae strains. Several major factors impacted on algae growth include carbon source, nitrogen source, phosphorous, sulfur, oxygen and other micronutrients.

### 2.1.3 Metabolic conditions of algae

Typically, microalgae may operate under three main types of metabolisms that include autotrophic, heterotrophic and mixotrophic. Different metabolisms

within a specific algal species may also shift from one type to another depending on the changes of environmental conditions (Mata, Martins and Caetano 2010). Species such as *Chlorella vulgaris, Haematococcus pluvialis, Spirulina (Spirulina) platensis, C. sorokiniana, Botryococcus braunii* and *C. zofingiensis* are reported well growth under all three metabolic conditions (Kim, et al. 2013). Many vital factors including organic carbon and substrate will impact the algae growth. Nutrients such as nitrogen and phosphorous, and factors such as pH, temperature, light intensity, salinity, and other operational parameters may also have a significant effect. These conditions and factors have an impact on algae growth and this contains cultivation types, nutrient concentration, biomass density, irradiance, medium components, growth temperature, dissolved oxygen, culture age and uniformity of mixing (Menetrez, 2012).

#### 2.1.3.1 Autotrophic (Phototrophic) Culture

Under autotrophic condition, microalgae capture sunlight and carbon dioxide from atmosphere and convert them into chemical energy through photosynthetic reactions and carbon source used in cellular functions.

The type of cultivation is usually set up as either an open pond system or photobioreactor at the industrial scale and the major advantage is low cost of the process. However, the biomass accumulation under autotrophic conditions may be limited by many factors such as available sunlight and temperature. Although, sunlight and carbon dioxide is free of cost, the relative high industrial processing operation cost is a big challenge of phototrophic microalgae technology.

#### 2.1.3.2 Heterotrophic Culture

Microalgae operating under strict heterotrophic conditions in the absence of light cannot fix carbon dioxide and they only use organic compounds such as carbon sources and energy to synthesize cell structure. Compared to phototrophic metabolism, heterotrophic cultivation has the benefits of (1) fast growth speed; (2) higher biomass production rate; (3) higher content of lipid in cells; (4) less water and land demand; (5) high-efficiency CO2 mitigation and (6) convenient harvesting (Zhen, et al. 2012; Gao, et al. 2010; Demirbas 2010). Heterotrophic cultured lipid productivities are reported to reach 20 times higher than those of harvested from autotrophic culture. The major shortcomings of heterotrophic cultivation ease of contamination and high cost of carbon and nitrogen compounds used for substrate.

#### 2.1.3.3 Mixotrophic Culture

As a combination condition of autotrophic and heterotrophic metabolism, mixotrophic cultivation may use either sunlight and inorganic carbon or organic carbon as their energy and carbon source, which takes advantage of altering environmental conditions. Cells synthesis requiring energy is taken from organic compounds and energy absorbed from light is converted into chemical energy then stored for further use. Based on the available light intensity and organic compound concentration, mixotrophic organisms have the possibility of living

under both autotrophic and heterotrophic conditions (Mata, Martins and Caetano 2010).

### 2.2 Biofuel production process and conversions

Algae biomass can be converted to several different types of possible biofuels and co-products depending on varying reaction conditions such as temperature, catalyst type, pressure and algal species used (Ghasemi, et al. 2012). Figure 2.2 is a summary of conversion from microalgae into different bio-products. And the production yield of algae biofuel is depending on the culture content, method, reactor and the reaction conditions (Menetrez 2012).

Biomass could contribute to about 38% of the direct fuel usage in the world by 2050 and 17% of electricity (Ghasemi, et al. 2012). Typically, biofuel is classified into three generations. First generation biofuel is defined as producing biodiesel and bioethanol from food crops such as sugarcane, corn, wheat and sorghum; nonfood crops for example, waste biomass, the stalks of wheat, corn and wood are selected as row sources to make cellulosic biofuels (gasoline) in second-generation biofuels production (Ghasemi, et al. 2012). And algae biofuels have been classified as third-generation biofuels that is regarded as the "advanced biofuels" (Ghasemi, et al. 2012) with obvious comparative merits over the other two generations including: (1) Growth rate of algae is high; (2) Algae can be cultivated in different environmental conditions; (3) Algae has the possibility of utilizing many kinds of water sources such as brackish coastal water, sea water and industrial waste water; (4) New technologies can be made

with use of a combination of waste treatment and algae biofuel production; (5) A large variety of algae species can be chosen for biofuel manufacturing (Menetrez 2012).

By 2025, the expected algal biofuel production will increase to 6 billion gallons (Thurmond 2009). Algal biomass has the possibility to be used directly as solid biofuel or may be converted into biogas, biohydrogen and other gaseous biofuel types (Ghasemi, et al. 2012).

### 2.3 Microalgae derived biodiesel

Biodiesel, with a chemical composition of fatty acid methyl esters, is regarded as an alternative diesel fuel with biodegradable, non-toxic and environmental friendly characteristics. Currently, most industrial produced biodiesel is derived from plant and animal oil. Biodiesel has been commercially produced since the 1960s (Chisti 2007). Soybeans are used as the major feedstock source of biodiesel production in United State (Chisti 2007).

Searching for the cheaper sources and waste sources from domestic, agricultural and industrial field is important to the biodiesel industry due to a desired decrease in capital cost and consumes the organic and inorganic pollutants from waste streams as well. These sources include, but are not limited to, corn, soybean, sugar cane leaves, food waste, microalgae and straw (Ha, Gang, et al. 2012). Table 2.2 indicates some of the reasonable biodiesel sources (Chisti 2007). In the United States, soybean is the most commonly used

source for biodiesel production, while canola oil, animal fat, palm oil, corn oil and waste cooking oil are also alternatives researchers are now paying more attention to (Felizardo, et al. 2006; Kulkarni and Dalai 2006). However, none of these sources have met the requirements of replacing the total amounts of fossil-based diesel consumed with the only exception of algae when comprehensively comparing all of the influential factors such as land area needed, production of dry biomass, time and oil content. Table 2.1 demonstrates that oil content between 20 and 50 % in different algae strains is a common level and can reach over 80 % by weight of dry biomass (Chisti 2007; Ghasemi, et al. 2012).

In the United States, ASTM standards for Biodiesel D6751 is the criterion to evaluate whether the biodiesel produced is qualified regardless of which feedstock was used during the production process. European standards are similar, but are executed through separate standards for vehicle oil and heating oil (Chisti 2007). The composition of microalgae biodiesel varies with several parameters mentioned below: (1) microalgae strains type; (2) carbon substrate and its concentration; (3) nitrogen substrate and its concentration; (4) growth conditions of lipid accumulation.

### 2.3.1 Transeasterification

There are four primary well-known methods to generate biodiesel: (1) Direct use and blending; (2) Microemulsions; (3) Pyrolysis; (4) Transesterification (Ma and Hanna 1999). Transesterification is most commonly

used for commercial production and is expected to continue for microalgae biodiesel production in the future.

Crude oil containing triglycerides reacted with methanol or alcohol is esterified with three molar fatty acid molecules and one molar glycerol molecule. Transesterification or alcoholysis is the major reaction for biodiesel production as shown in Figure 2.1 for making methyl esters of fatty acids (biodiesel). The reaction for transesterification of triglyceride with alcohol is performed as several consecutive reactions and is summarized in Figure 2.1 (a, b). The first step is triglycerides converted to diglycerides, then followed by the reactions of diglycerides converted to monoglycerides and finally the conversion of monoglycerides to glycerol and fatty acid methyl esters (FAME) (Fukuda, Kondo and Noda 2001). Excess methanol (5 to 6 moles) is fed to react with each mole of triglyceride to guarantee this process is driven in the direction of producing methyl esters (Chisti 2007) in industrial applications. The excess methanol should then be recovered when the reaction is complete and used for further processing.

### 2.3.2 Catalysts for transesterification

Transesterification can be catalyzed by three types of catalysts, which include acids, alkalis and lipase enzymes. The most widely applied catalysis is alkali-catalyzed transesterification using sodium and potassium hydroxide due to faster reaction velocity and lower commercial price (Fukuda, Kondo and Noda 2001). Compared with alkali-catalyzed transesterification, lipase enzymes have

performed well; however, the relative high cost has restricted common application of these catalysts (Fukuda, Kondo and Noda 2001).

Acids widely used for transesterificaiton process contain sulfonic (Guerreiro, et al. 2006), sulfuric acids (Canakci and Gerpen 2001), phosphoric and hydrochloric (Fukuda, Kondo and Noda 2001). Acid-catalyzed transesterifications reactions are slow which require over 100°C and 3 hours to finish the conversion process (Freedman, Pryde and Mounts 1984). However, it has been investigated that acid-catalyzed transesterifications can achieve high free fatty acid and water concentrations and this characteristic makes acid-catalyzed transesterifications more suitable for glycerides production (Fukuda, Kondo and Noda 2001).

Alkali-catalyzed transesterification is the most common type used in biodiesel production since it can catalyze the reactions much faster (over 4000 times) than acid-catalyzed method (Fukuda, Kondo and Noda 2001). The alkali catalyst such as NaOH (Wang, et al. 2007), KOH (Noiroj, et al. 2009), carbonates sodium methoxide, sodium propoxide and sodium butoxide can be found to use for transesterifications (Fukuda, Kondo and Noda 2001).

There are some general disadvantages of all chemical transesterification catalyst such as their high energy demands and many difficulties appeared during glycerol recovery (Fukuda, Kondo and Noda 2001). Moreover, the reacted wastewater for acid and alkali catalyst should specific treatment due to the acidic and alkaline compositions contained it. Biological catalyst – enzymes have the possibility to overcome or diminish the bad effects mentioned above. Many lipases can be chosen to catalyze transesterification process, while bacteria and

fungi lipases are the most commonly used (Gaurav, Srivastava and Singh 2013). Lipases enzymes for biodiesel production can reduce the steps that results in decreasing of energy and cost. Another advantage includes saving waster decreasing the capacity of wastewater required for further treatment. However, enzyme-catalyzed transesterification has a main drawback that the reaction rate is relatively low and the enzymes may become inactive by processing factors (Shah, Sharma and M.N.Gupta 2003).

### 2.3.3 Alternative potential feedstock for microalgae biodiesel production

The greatest challenge for microalgae biodiesel production industry is the price. Many researches now are beginning to focus on developing more economical cultivated medium for algal biofuels. Heterotrophic and mixotrophic conditions had been proven that were much better than autotrophic condition for algae growth and lipid accumulation. However, they required organic carbon (e.g. glucose), nutrients like nitrogen (e.g. yeast extract) and phosphorous, and also enough water for cells growth which account for around 80% of the total medium costs (Li, Xu and Wu 2007).

#### 2.3.3.1 Traditional feedstock for biodiesel

Generally speaking, any biological materials that can produce oils have the potential to create biodiesel in either direct or indirect ways. Rudolph Diesel was the original diesel engine that was invented to use diesel fuel with a

compression ignition engine and the inventor tried to supply peanut oil to the engine at the very beginning (Alper 1990; Gaurava, Srivastava and Singh 2013).

Vegetable oil is a major source utilized in biodiesel field and many types of vegetable oils have been reported in previous studies like palm oil (Al-Widyan and Al-Shyoukh 2002), soybean oil (Cao, Han and Zhang 2005), sunflower oil (Antolín, et al. 2002), corn oil (Patil and Deng 2009), coconut oil (Jitputti, et al. 206), canola oil (Ma and Hanna 1999), Jatropha oil (Tiwari, Kumar and Raheman 2007), restaurant waste oil (Canakci 2007) and coffee oil (Oliveira, et al. 2008).

#### 2.3.3.2 Possible waste sources used as feedstock

Since microalgae growth requires different and a large amount of carbon, nutrients including nitrogen and phosphorous, many types of industrial wastewater with a relative high concentrations of them can be considered to support microalgae reproducing (Suali and Sarbatly 2012) in recent years. In addition, a combination of microalgae biofuel and wastewater treatment is getting more and more attentions because its win-win strategy for both environment and economic. The history of microalgae wastewater treatment has been improved for a long time. Though, compared with the traditional wastewater treatment methods, applications of microalgae in this area is still limited by many factors like capital cost and the facilities, more and more researchers have put an eye on this innovated method (Pittman, Dean and Osundeko 2011).

The waste from brewer fermentation broth is a potential resource for the biofuel production since this kind of waste is abundant in proteins, water, dead yeast cells, nutrients such as nitrogen and phosphorus and enzymes that can be supplied enough carbon, nitrogen to algae growth. The waste brewer fermentation has been studied as an economical culture medium for bio-ethanol production in several researches (Ha, et al. 2012; Ha, Shah, et al. 2011). Studies on growing algae on dairy and municipal wastewater and lipid production for biofuel have been reported too (Woertz, et al. 2009). Woertz indicated in his experiment that the peak lipid content range from 14-29% and the maximum lipid productivity for the municipal wastewater reached 24 mg/day/L. The ammonium and orthophosphate in wastewater were removed by >99%. This study demonstrated municipal wastewater as a potential feedstock for biofuels production.

### 2.3.4 Other microalgae biofuel potential applications

#### 2.3.4.1 Bio-ethanol

Bioethanol is another well-known biofuel that can be treated as fossil-derived petrol alternatives as biodiesel discussed above.

Cellulose and glycoproteins are built cell walls and in some algae species, the cell walls contain more than 50% of starch (Ghasemi, et al. 2012). These years, some innovated technologies attempt to use hydrolyzed sugars from cellulose and hemicellulose to convert a larger amount of biomass into ethanol. It

is demonstrated that nearly 5000-15000 gallons of bioethanol/acre/year are derived from algae and compared to traditional corn starch sources and this productivity meets approximately 10 to 30 times higher than that of corn starch (Ghasemi, et al. 2012). Biobutanol is a comparable automotive fuel form with bioethanol and it can be created using the same feedstocks and similar production process as bioethanol (Ryan 2009). However, biobutanol performs better than bioethanol in not only lowering vapor pressure which results in decreasing the evaporative emissions but also increasing the outlet energy density (Ghasemi, et al. 2012).

#### 2.3.4.2 Biohydrogen

The most significant improvement of hydrogen fuel is no NOx emissions and water is the only exhaust product during this process. In addition, since the production of hydrogen is in gas phase, it can be released in a short time while not accumulates in the medium to poison the cells. Hydrogen is usually produced in industrial field by steam reformation of fossil fuel (Ghasemi, et al. 2012). The other common ways of hydrogen generation are photolysis of water by specific microalgae and fermentation of organic compounds and materials under dark or light conditions.

The biohydrogen generation was first recorded before over 65 years ago by researching on Scenedesmus obliquus, a green algal species (Das and Veziroğlu 2001). Nitrogenase and hydrogenase are two major required enzymes in biohydrogen engineering by photosynthetic algae. Cells grow on the first stage

and then hydrogen evolves on the second stage (Figure 2.3). Under anaerobic condition, some algae species can produce hydrogen by reacting water and nature light and this can be classified into direct biophotolysis and indirect biophotolysis. Algae species that have been studied for biohydrogen yield including Chlamydomonas reinhardtii, Chlorococcum littorale, Platymonas subcordiformis, Anabaena, Nostoc muscorum, N. spongiaeforme, Westiellopsis prolifica, Oscillotoria Miami BG7 and Aphanothece halophytico (Ghasemi, et al. 2012).

#### 2.3.4.3 Biomethane

Another primary pathway of algae biomass conversion is called anaerobic digestion that produces methane, carbon dioxide, hydrogen, hydrogen sulphide and ammonia. This process can be summarized into four steps – Hydrolysis, Acidogenesis, Acetogenesis, Methanogenesis (Figure 2.4). Carbon nitrogen ratio is the major limited parameter of biogas productivity. In industrial field, the feedstock of anaerobic digestion is always required to meet a 25-30 carbon nitrogen ratio and this is the reason that waste water is considered as a suitable feedstock for biomethane (Ghasemi, et al. 2012). However, biomethane from algae is not widely accepted at present because of its much higher capital cost than crops.

### 2.4 Microalgae biofuel economic analysis

The cost of microalgae-based biofuel is the most important aspect to evaluate whether this type of energy can be large scaled used in future. An overview of algae biofuel economic analysis is focusing on the harvesting cost that includes drying algae, equipment capital cost and maintenance fee, chemicals used in biofuel production process, electricity and labor force for all the process (Ghasemi, et al. 2012). Algal oil production cost may vary with many factors such as the biomass yield, lipid content and cultivation method (Menetrez 2012). There has a generally used equation indicated the competitive substitute of algal biofuel for petroleum diesel (Chisti 2007):

$$C_{algal\ oil} = 6.9 \times 10^{-3} C_{petroleum}$$

$C_{algal\ oil:}$ the price of microalgae oil in dollars per liter
$C_{petroleum}$: the price of crude oil in dollars per barrel

Environmental impacts should be also considered when model the biofuel analysis system. Dominant aspects existed in previous literatures are including water resources, land use and location, nutrient and fertilizer use, carbon fertilization, fossil fuel inputs, eutrophication, algae toxicity and et al. (Slade and Bauen 2013).

**Tables and Figures**

Table 2.1 Oil content of different microalgae strains

| Microalgae | Oil content (% dry wt) | Microalgae | Oil content (% dry wt) |
|---|---|---|---|
| Ankistrodesmus | 28-40 | Hantzschia species | 66 |
| Anabaena cylindrica | 7-Apr | Isochrysis galbana | 21.2 |
| Botryococcus braunii | 25-75 | Isochrysis sp. | 25-33 |
| Chaetoceros muelleri | 33 | Monallanthus salina | 20-22 |
| Chlamydomonas species | 23 | Nannochloris sp. | 20-35 |
| Cllorella emersonii | 25-63 | Nannochloropsis sp. | 31-68 |
| Chlorella minutissima | 57 | Neochloris oleoabundans | 35-54 |
| Chlorella protothecoides | 14-57 | Nizschia sp. | 45-47 |
| Chlorella sorokiniana | 22 | Pavlova lutheri | 35 |
| Chlorella sp. | 28-32 | Phaeodactylum tricornutum | 20-30 |
| Chlorella vulgaris | 14-56 | Prostanthera incisa | 62 |
| Crythecodinium cohnii | 20 | Prymnesium parvum | 22-39 |
| Cylindrotheca sp. | 16-37 | Scenedesmus dimorphus | 16-40 |
| Dunaliella primolecta | 23 | Schizochytrium sp. | 50-77 |
| Dunaliella tertiolecta | 36-42 | Skeletonema costatum | 13-51 |
| Euglena gracilis | 14-20 | Stichococcus species | 33 |
| Ellipsoidion sp. | 27 | Tetraselmis sueica | 15-23 |
| Haemotococcus pluvialis | 25 | Zitzschia sp. | 45-47 |

Source: Adapted from Chisti 2007, Ghasemi, et al. 2012 and Menetrez 2012

Table 2.2 Comparison of some reasonable biodiesel sources

| Crop | Oil yield (L/ha) | Land area needed (M ha)[a] | Present of existing US cropping area[a] |
|---|---|---|---|
| Corn | 172 | 1540 | 846 |
| Soybean | 446 | 594 | 326 |
| Canola | 1,190 | 223 | 122 |
| Jatropha | 1,892 | 140 | 77 |
| Coconut | 2,689 | 99 | 54 |
| Oil palm | 5,950 | 45 | 24 |
| Microalgae[b] | 136,900 | 2 | 1.1 |
| Microalgae[c] | 58,700 | 4.5 | 2.5 |

[a] For meeting 50% of all transport fuel needs of the United State.

[b] 70% oil (by wt) in biomass.

[c] 30% oil (by wt) in biomass.

Source: Adapted from Chisti 2007

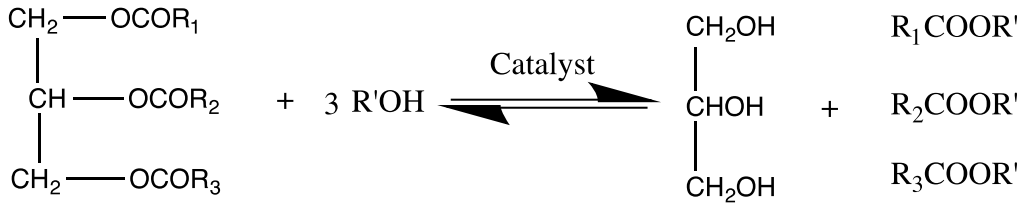


Figure 2.1 (a) Transesterification of triglycerides

Source: Adapted from Fukuda, Kondo and Noda 2001.


Figure 2.1 (b) Three consecutive and reversible reactions of transesterification process

Source: Adapted from Fukuda, Kondo and Noda 2001.

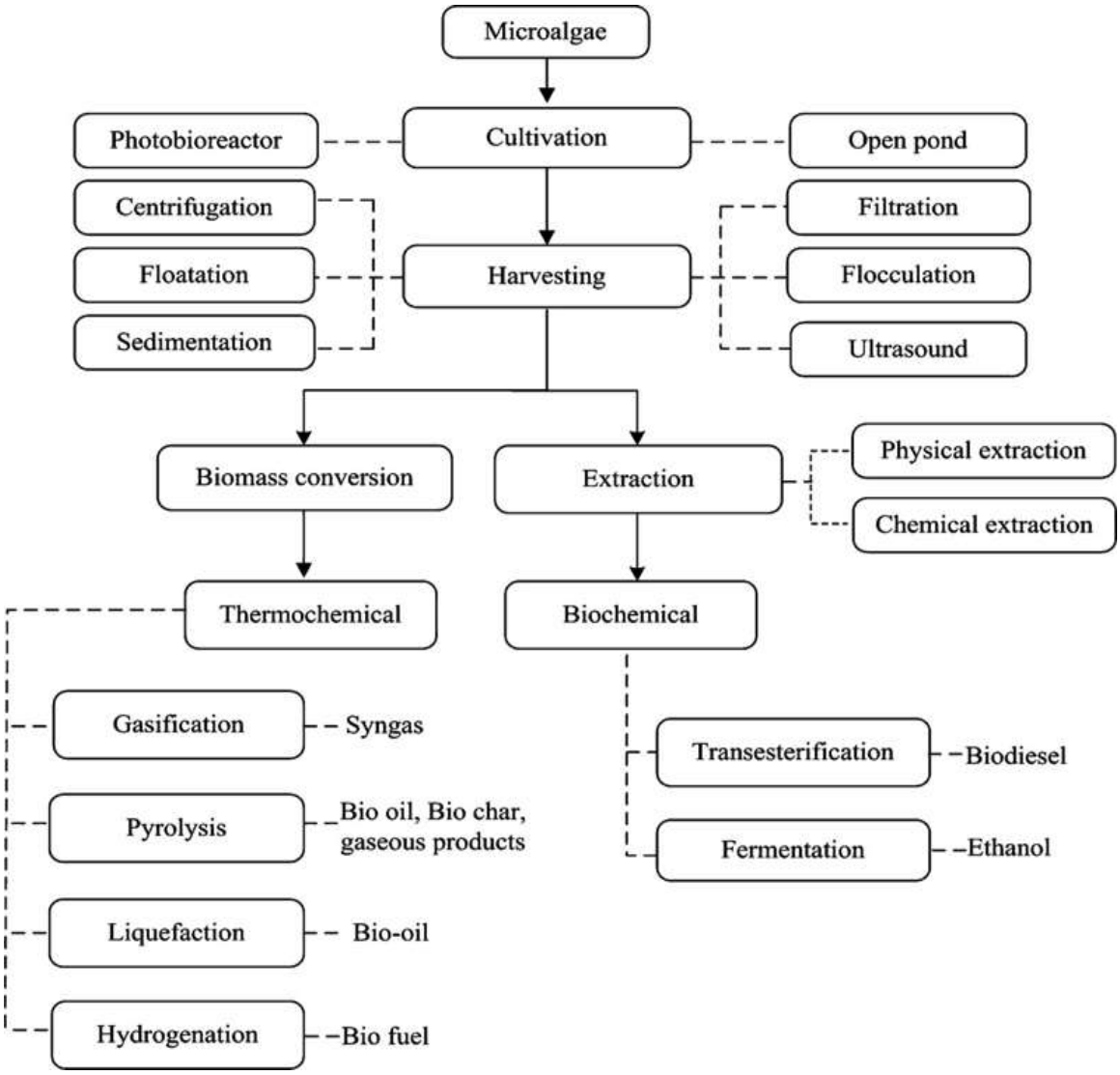


Figure 2.2 Conversion of microalgae into bio-products

Source: Suali and Sarbatly 2012

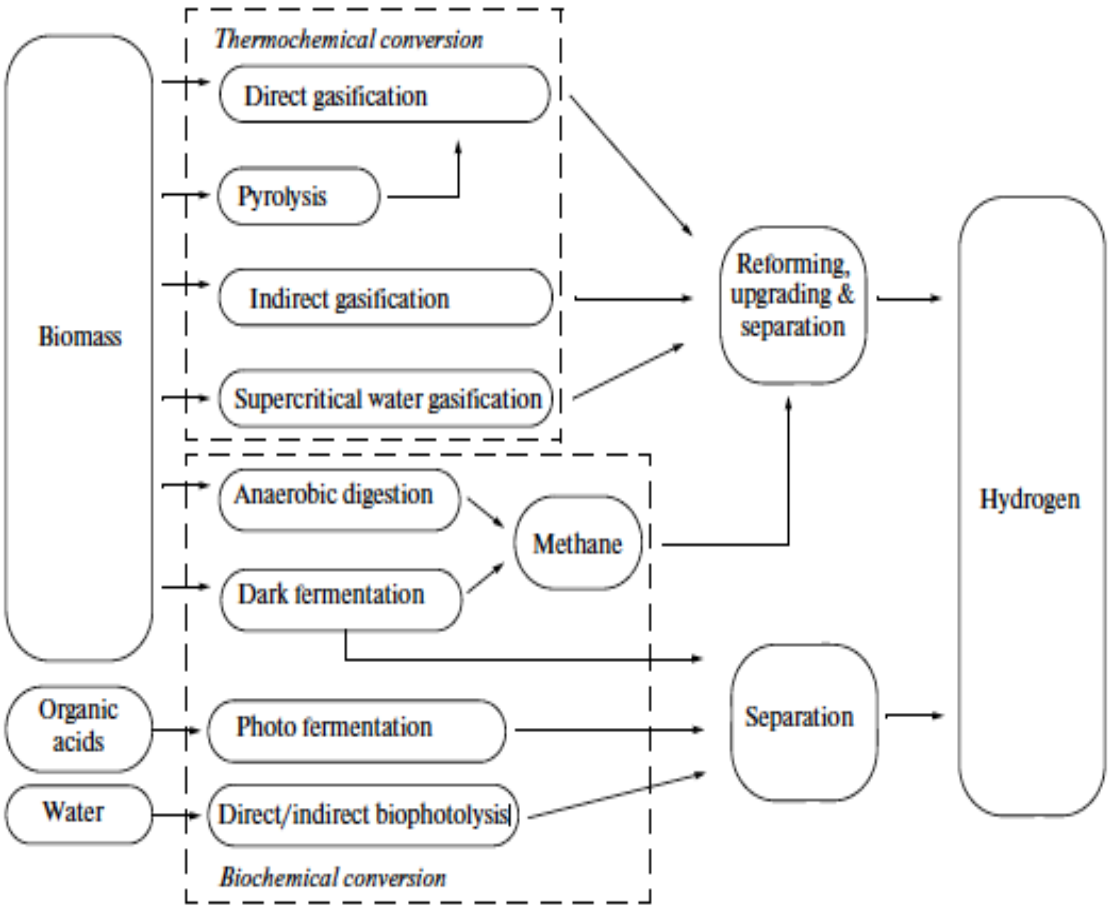


Figure 2.3 Microalgae biohydrogen pathways

Source: Ghasemi, et al. 2012

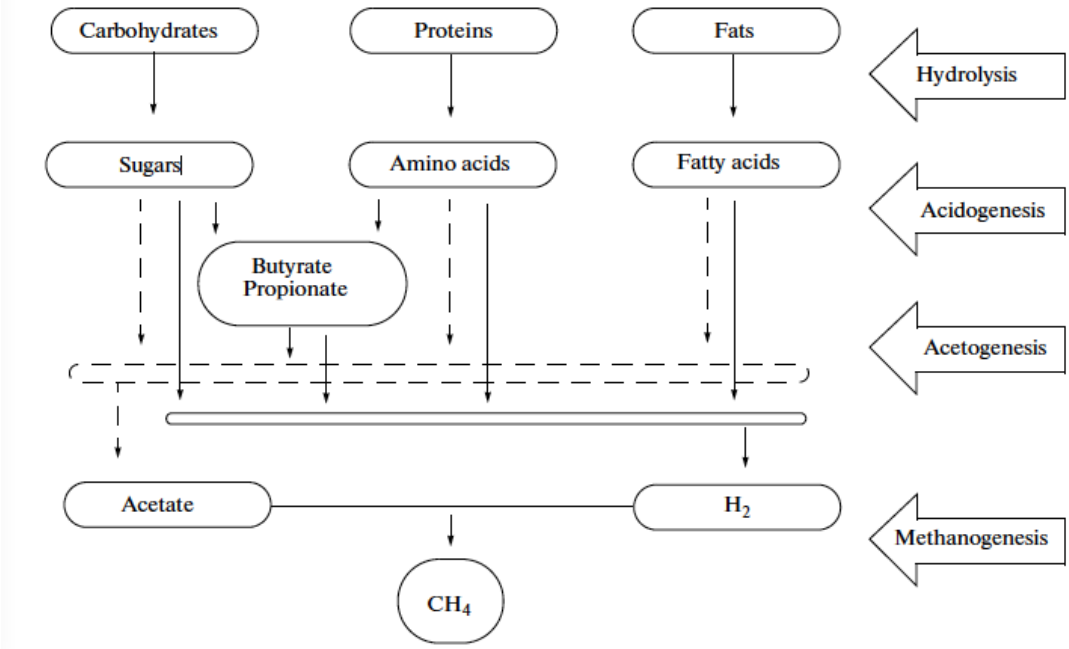


Figure 2.4 Biomethane from anaerobic digestion

Source: Ghasemi, et al. 2012

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# CHAPTER III

# BIOMASS AND LIPID PRODUCTION OF CHLORELLA PROTOTHECOIDES UNDER HETEROTROPHIC CULTIVATION ON A MIXED WASTE SUBSTRATE OF BREWER FERMENTATION AND CRUDE GLYCEROL

## 3.1 Introduction

In the past decade, global warming and energy deficiency are two worldwide issues in modern society. The United States Energy Information Administration (EIA) has predicted that non-renewable energy such as fossil fuel may be depleted or economically nonviable by humanity in the next 90 years (Sivakaminathan 2012). Biodiesel, with a chemical composition of fatty acid methyl esters (FAME) and derived via transesterification is regarded as an alternative of diesel fuel due to its biodegradable, non-toxic and environmental friendly characteristics (Michigan Department of Technology, Management & Budget n.d.). Biodiesel has been commercially produced since the 1960s (Chisti 2007). Soybeans are used as the major feedstock source of biodiesel production in United State (Chisti 2007). However, traditional biodiesel, mostly using grain or soybean as its basic materials, requires large plant area and may come up with the problem of competing with human food (Heredia-Arroyo, Wei and Hu 2010). Microalgae derived biodiesel —the third generation of biodiesel, with the advantages of being grown on non-arable land, in fresh water, or even in

seawater and fast accumulated within a short time— is considered as a promising biodiesel production method (O'Grady and Morgan 2011).

There are so many algal species that can be used to produce biodiesel and *Chlorella protothecoides* is one of the most studied. *Chlorella protothecoides* have been demonstrated to grow both autotrophically and heterotrophically. Further, *Chlorella* microalgae is one of the most understood species in research field compared with various other algae strains. *Chlorella protothecoides* have been reported in many publications and achieved a relative high biomass and lipid accumulation using different carbon sources and under several environmental conditions (Chen and Walker 2011; Miao and Wu 2006; Xu, Miao and Wu 2006; Heredia-Arroyo, Wei and Hu 2010; O'Grady and Morgan 2011).

The greatest challenge for the microalgae biodiesel production industry is the price. Heterotrophic and mixotrophic conditions had been proven to be much better than autotrophic condition for algae growth and lipid accumulation. However, they required organic carbon (e.g. glucose), nutrients like nitrogen (e.g. yeast extract) and phosphorous, and also enough water for cells growth which account for around 80% of the total medium costs (Li, Xu and Wu 2007). Many researches studies are beginning to focus on developing more economical cultivated substrates for algal biofuels. Due to the large amount requirements of different nutrients by algae, many types of industrial wastewater with a relative high concentration of nitrogen, most in terms of the individual amino acids (Suali and Sarbatly 2012), carbon and other utilizable elements have been becoming a considerable biodiesel feedstock in recent years. A combination of microalgae

biofuel and wastewater treatment is getting more and more attentions because it is a win-win strategy for both the environment and economics (Cabanelas, Arbib, et al. 2013; Cabanelas, Jésus, et al. 2013).

The price of crude glycerol, a primary by-product of biodiesel production, has decreased in past years due to the large increasing of its productivity and cheaper purification cost (Chen and Walker 2011). This makes the crude glycerol to be a more competitive carbon substrate than sugars. Based on the analysis of other previous studies (Thompson and He 2006), different feedstocks and biodiesel production conditions are two main factors that can impact the composition of crude glycerol (Chen and Walker 2011).

The brewer fermentation process has been developed and well established for a very long time. Water, starch source, a brewer's yeast and a flavoring (hops) were regarded as basic ingredients for beer production (Ha, Shah, et al. 2011), and through fermentation, sugar and glucose were converted to bio-ethanol by specific bacteria and yeast. Brewer fermentation waste collected from the last step of the beer industrial process attempts to be used as an alternative substrate to support algae growth because of its higher concentration of nitrogen. Some studies believed that most of the nitrogen left in brewer fermentation waste is coming from the residue yeast cells (Ha, Shah, et al. 2011).

Therefore, the treatment and utilization of mixed brewer fermentation waste (the major nitrogen source) and crude glycerol waste (the major carbon

source) seems to possibly meet the supplement requirements of microalgae and seems suitable from economic and environmental friendly point of view.

## 3.2 Material and Methods

### 3.2.1 Microorganism and culture medium

All chemicals used in this experiment were obtained commercially from authentic sources and of analytical grade.

*Chlorella protothecoides* UTEX 256 algae used in this experiment was purchased from the Culture Collection of Algae at the University of Texas (Austin, TX). The components of a modified basal medium are prepared as follows (per liter): 0.7 g $KH_2PO_4$, 0.3 g $K_2HPO_4$, 0.3 g $MgSO_4 \cdot 7H_2O$, 25 mg $CaCl \cdot 2H_2O$, 25 mg NaCl, 3 mg $FeSO_4 \cdot 7H_2O$, 0.01 mg Vitamin B1 and 1 ml A5 solution (Chen and Walker 2011; Chen and Walker 2012). *Chlorella protothecoides* microalgae cells were suspended in the basal medium with supplement of 30 g/L pure glucose and 4 g/L yeast extract (50 wt% as total organic carbon and 15 wt% as total nitrogen) and this was used for algae growth as a control group (BM-GY). The substrate used for waste group were prepared by mixing brewer fermentation waste (Stock I in table 3.1 with 2.48 g/L total nitrogen and 26.38 g/L total organic carbon) and crude glycerol (Stock I in table 3.2 with 30.55 wt% total organic carbon) to obtain the equivalent quantities of initial concentration of total organic carbon and total nitrogen as in control group. An old *C. protothecoides* strain (room temperature at 28 °C for 6 months) was used in

batch 1 and a new *C. protothecoides* strain (purchased from University of Texas and cultured immediately) was used in batch 2 and batch 3 in both of these two groups.

### 3.2.2 Cultivation methods

The heterotrophic batch cultures were carried out in 500 ml shake-flasks containing 200 ml of the culture medium. The shake-flasks were covered by aluminum foil to block out any light outside the system. All medium were autoclaved at 121 °C for a 15-20 minute cycle prior to inoculation. The initial pH of the medium was adjusted to 6.8 using 0.5 M HCl and 0.5 M KOH. The cultures temperature was kept at 28 °C with shaking at 220 rpm to guarantee enough air transportation within the flasks during a 7-day cycle. The exponentially growing *Chlorella protothecoides* under heterotrophic condition from glucose and yeast extract substrate cultures were inoculated for further batch experiments. The inoculums size was at 10 % (volume/volume). Three batches (batch 1, 2, and 3) with triplicate treatments were maintained for each medium source (BM-GY group and mixed waste group).

### 3.2.3 Brewer fermentation waste characterization and pre-treatment

The brewer fermentation waste stream was collected at the end of the brewer fermentation step from Biosystems Engineering Lab of Clemson University. Since this kind of waste contains wet solid sediment, pre-treatment is

needed to prepare the stock. Fermentation waste was shaken by hand to break large sedimentary particles and then heated to nearly 100 °C with continuous stirring and temperature control to remove most of the volatile components such as alcohol. A Polytron® shear mixer (PT 1200 C, Kinematica AG) was applied to homogenize and shear the yeast and bacteria cells in waste for 10 minutes to obtain more soluble nitrogen, carbon and other usable elements and the waste was then filtered by a liquid process filter. The filtered pre-treated fermentation waste was autoclaved and refrigerated at 4 °C. Based on the analysis using a Shimadzu TOC-V, TMN-1 instrument of fermentation waste, the stock filtrated brewer fermentation waste (Stock I in Table 3.1 was pre-treated and used as substrate in this study) was reported as containing 2.48g/L as total nitrogen and 26.38g/L as total organic carbon. The elemental compositions of three individual brewer fermentation waste samples were shown in Table 3.1 and this was determined according to the wet ash digestion procedure from Agricultural Service Laboratory of Clemson University (Clemson, USA) by an inductively coupled plasma (ICP) method.

### 3.2.4 Crude glycerol characterization and pre-treatment

The crude glycerol was obtained from the Clemson University Sustainable Biodiesel Lab. This biodiesel by-product mainly constituted by excess methanol, water and glycerol by-product (Kiss and Ignat 2012) was derived from alkali-catalyzed transesterification of oil with methanol using potassium hydroxide.

To purify and concentrate the crude glycerol, the biodiesel waste was

heated on a hot plate to 70 °C for 15 minutes to remove excess methanol. To adjust the pH under 3, sulfuric acid was added into the waste while stirring during this process to guarantee the reaction within the waste goes to completion. The mixture was centrifuged under 3000 rpm for 10 minutes creating three layers that included biodiesel, glycerol, and soap from top to bottom isolated by density differences after the centrifuge. The soap solid layer can be easily removed from the mixture and biodiesel and glycerol were transferred into a separatory funnel to separate by gravity. After the pre-treatment, the crude glycerol was autoclaved and stored for further experiments. The total organic carbon of the stock crude glycerol was analyzed using a Shimadzu TOC-V, TMN-1 instrument that was shown to contain 30.55% of total organic carbon (Stock I in Table 3.2 was pre-treated and used in this study). Total nitrogen was found below the accurate detectable amounts and was considered insignificant. The elemental characteristics of three individual crude glycerol samples were performed with an inductively coupled plasma (ICP) method in accordance with the wet ash digestion procedure from Agricultural Service Laboratory of Clemson University (Clemson, USA) and are shown in Table 3.2.

### 3.2.5 Analytical procedures

$OD_{540}$ values were measured as an indicator of cell growth. Data of optical density was correlated with cell dry weight to obtain accurate results. The fermentation waste and crude glycerol stock samples were sent to Agricultural

Service Laboratory of Clemson University for elemental analysis by an inductively coupled plasma (ICP) method. Total nitrogen and total organic carbon of substrates and their changes during the growth phase were diluted with DDI water below 25 ppm and measured by a Shimadzu TOC-V, TMN-1 instrument. Potassium hydrogen phthalate and potassium nitrate were used to prepare TOC standards and TN standards, respectively. The cells dry weights were obtained by centrifuging the sample at 3,000 rpm for 15 min and drying them in an oven for 48 hrs at 80 °C. Lipid content was determined by a modified method from previous studies that were using hexane to extract the biomass (Chen and Walker 2011). All samples were collected every 24 hrs.

Data analysis involved scatter plots, regression analysis, and t-test. All calculations were performed using JMP Software from Statistical Analysis System (SAS, SAS Institute, USA). All statistical significance test were based on $\alpha$=0.05.

### 3.3 Results and Discussion

#### 3.3.1 Analysis of the composition of fermentation waste and crude glycerol from biodiesel waste

The feedstocks used in this research are a mixture of waste steam obtained from two different industrial producing processes. Since these waste medium sources were collected from downstream processes, the composition and characteristics of the waste varied with many factors such as the raw materials, volume, production quality and process. The ICP elemental analysis of

brewer fermentation waste and crude glycerol were summarized in Table 3.1 and Table 3.2 and the stocks shown in these tables were sampled independently from three separated batches, respectively. Stock I in both of the tables was used as the target waste stocks to prepare the growth medium for *C. protothecoides*.

Potassium, phosphorus, sulfur and magnesium were the major elements in brewer fermentation, while potassium and sodium were dominant elements in crude glycerol. Other required trace elements such as aluminum, calcium, iron, copper, manganese and zinc were detected to contain in brewer fermentation and crude glycerol too with suitable concentrations for algae reproduction. Some studies believed that these trace elements had significant impacts on algae growth and lipid accumulation. Iron impacts on biomass and lipid production of marine microalgae species *Chlorella vulgaris* have been studied and was reported that *C. vulgaris* could accumulate higher lipid content in the medium with $1.2 \times 10^{-8}$ mol/L $FeCl_3$ than without $FeCl_3$ (Chen and Walker 2011). Other factors including nitrogen starvation, decreasing phosphorus concentration, silicone deficiency, and supplement of cadmium were investigated to effect lipid accumulation in some algae species (Reitan, Rainuzzo and Olsen 1994; Lynn, et al. 2000; Guschina and Harwood 2006).

However, the comparisons of different elements within three individual batches for both fermentation waste and crude glycerol in Table 3.1 and Table 3.2 demonstrated that the concentrations of each element from different batches could vary with a large range. In Table 3.1, the concentration of calcium of brewer fermentation waste in Stock I and Stock II were 28.32 ppm and 35.53 ppm, respectively, while, this concentration reached 180.25 ppm in Stock III

which was over 5 times greater than the other stock solutions. Similar results may be certified by contrasting the data of iron, aluminum and copper from the three stocks. Significant fluctuations of elemental concentrations from the waste of independent batches were observed in glycerol. As shown in Table 3.2, Stock II and Stock III of crude glycerol contained 36,236.1 ppm and 31,375.5 ppm potassium, respectively, however, potassium concentration in Stock I was only 3,535.87 ppm. The high potassium concentrations in crude glycerol samples were due to the alkali-catalyzed transesterification process.

Table 3.1 and 3.2 indicate that brewer fermentation waste is a good resource of nitrogen and crude glycerol could supply enough organic carbon for algae. At the meantime, the mixture of these two wastes seems to contain enough other required elements of cell growth. Preparing stocks using waste streams should take the large variation of elemental concentration in individual batch into consideration in further research.

### 3.3.2 Effect of the old and new strains in BM-GY and mixed waste substrates on biomass and lipid accumulation in batch fermentation of *Chlorella protothecoides*

#### 3.3.2.1 Biomass and lipid accumulation

To test the possible use of a waste mixture collected from brewer fermentation and biodiesel production downstream as carbon and nitrogen sources for *C. protothecoides*, batch fermentation experiments without pH and dissolved oxygen control were designed. The total organic carbon and total nitrogen in the control group were 14 g/L as C and 0.6 g/L as N. The waste group

was calculated and prepared using the analyzed data of fermentation waste Stock I and crude glycerol waste Stock I to meet the equivalent amount of initial total organic carbon and total nitrogen concentration as control group. Figure 3.1 shows the biomass concentrations in three batches of BM-GY and waste substrates over a 7-day fermentation cycle. As shown in Figure 3.1, the biomass increased at a high rate on the first two days in BM-GY substrate group and then gently accumulated from day 3-6. At day six, the biomass concentrations of old strains (batch 1) and new strains (batch 2 and 3) in BM-GY group were 14.47 g/L and 11.43 g/L, respectively. Both old strains and new strains cell growth speeds in waste medium were lower than those of BM-GY in day 1 and day 2, from day 3-7, the biomass kept continuously increasing to give the concentrations of 14.07 g/L in old strains batch and 12.73 g/L in new strains batch on the sixth day (Figure 3.1).

Based on the data analysis of day 5-7, there is no significant difference in the mean biomass accumulation between two medium groups in the old strains batches (day 5 t-test, p-value=0.1338; day 6 t-test, p-value=0.4196; day 7 t-test, p-value=0.4573). However, the mean biomass concentrations of the new strains batches within BM-GY group and waste group were significantly different (day 5 t-test, p-value=0.0498; day 6 t-test, p-value=0.0073; day 7 t-test, p-value=0.0061). These results suggested that the new strains accumulated more biomass in the waste medium. The results also indicate that in both of these two substrates, on the sixth day and seventh day the biomass concentrations of the old strains were significant higher than those of the new strains (BM-GY: day 6 t-

test, p-value=0.0003; day 7 t-test, p-value=0.0002) (Waste: day 6 t-test, p-value=0.0157; day 7 t-test, p-value=0.0016).

Figure 3.2 summarizes the trends of lipid concentration in the BM-GY group and waste group of three batches. In the BM-GY group, the lipid concentration achieved 6.33 g/L in the old strains batch and 5.81 g/L in the new strains batch on day 6, while the lipid concentrations of old strains and new strains in mixed waste group found on the sixth day were 5.97 g/L and 6.57 g/L, respectively. In figure 3.3, the mean lipid content (g lipid/g biomass) in all bathes did not show significant differences (t-test, p-value=0.8070) between BM-GY group and waste group during a fermentation period. However, the comparison of old strains batch and new strains batch shows that in both of the two different substrate groups, the mean lipid contents of the new strains batch were significant higher than those of the old strains batch (t-test, p-value<0.0001).

Commonly, algae cells can accumulate their lipid content up to 60-70% due to nitrogen deficiency (Chen and Walker 2011). In this study, the lipid contents were kept stable within the range of 47.0% (w/w)-50.6% (w/w) and were not dramatically influenced by the two different substrates in this study, however, the new strains performed better than the old strains in lipid accumulation in BM-GY group and waste group.

#### 3.3.2.2 Total organic carbon and total nitrogen consumption

Total organic carbon was consumed with the biomass accumulation during the 7-day fermentation period. As shown in Figure 3.4, the final

concentrations of total organic carbon of the old strains batch and new strains batch in BM-GY group were 1.03 g/L and 1.66 g/L, respectively. And in the waste group, the total organic carbon concentrations at the seventh day of the old strains batch and new strains batch achieved 1.94 g/L and 3.36 g/L, separately. Significant differences were observed between the means of the old strains batch and the new strains batch from day 1 to day 2 in the BM-GY group (day 1 t-test, p-value=0.0072; day 2 t-test, p-value=0.0046) and day 1 to day 3 in the waste group (day 1 t-test, p-value=0.0393; day 2 t-test, p-value=0.0268; day 3 t-test, p-value=0.0026). From day 4-7, the concentrations of total organic carbon of both the old strains batch and new strains batch in the waste group were significant higher (t-test, all p-values<0.05) than those in the BM-GY group which may indicate that the mixed waste medium contained more organic carbon that was not utilized by *C. protothecoides*.

The trends of total nitrogen consumption of BM-GY group and mixed waste group were quite similar (Figure 3.5). The most significant consumption of nitrogen was detected on the first day in both old strains batch and new strains batch and then the concentrations of total nitrogen slightly changed between day 2 and day 7 in old and new strains batches within the two different substrates. There were no significant differences in the means of total nitrogen were observed between old strains batch and new strains batch (t-test, all p-values>0.05) and the mean amounts of total nitrogen consumption in the two substrates were analyzed with no obvious differences too (t-test, all p-values>0.05).The final total nitrogen concentration of BM-GY group was 0.12 g/L in old strains batch and 0.20 g/L in new strains batch. The concentrations of total

nitrogen in the waste group were achieved 0.16 g/L in the old strains batch and 0.20 g/L in the new strains batch.

*Chlorella protothecoides* has shown a good adaptability of growing in brewer fermentation waste and biodiesel crude glycerol. Brewer fermentation waste was shown to be a potential nitrogen source and parts of carbon source too. At the same time, the results also gave convincing evidence of feeding crude glycerol as a carbon source for algae growth. Several publications have referred to using glycerol as an alternative carbon source to improve lipid production and decrease the capital cost (Chen and Walker 2011; O'Grady and Morgan 2011; Heredia-Arroyo, Wei and Hu 2010; Cabanelas, et al. 2013). In addition, the results displayed in this study showed that 81.5 wt% of total organic carbon (mean value of three batches) and 65.1 wt% of total nitrogen (mean value of three batches) within the mixed waste group were removed by algae which indicated there was a possibility of combining biofuel production and industrial waste water treatment in the future. There have already been some research topics focused on treating wastewater by algae and lowering the organic compounds such as carbon and nitrogen in the meantime (Kothari, et al. 2013; Mitra and Lamsal 2012; Ha, Shah, et al. 2011; Cabanelas, et al. 2013).

### 3.3.3 Model of the relationship between optical density (OD) and algae biomass and the discussion of its statistical reliability

Generally, optical density, also defined as absorbance, at specific wavelengths is widely applied as an easy and obvious method to reflect and

monitor the cells growth and biomass accumulation in most algal studies (Mitra and Lamsal 2012; Kothari, et al. 2013; Chen and Walker 2011; Cabanelas, Arbib, et al. 2013; Heredia-Arroyo, Wei and Hu 2010; Wang, et al. 2013; Xu, Miao and Wu 2006; Gao, et al. 2010; Kim, et al. 2013). Figure 3.6 reveals the linear statistical model approach of the relationship between optical density and biomass concentration. Statistical analysis of batches 1, 2 and 3 of two tested substrates were shown as Equation (I), Equation (II) and Equation (III), respectively.

$$y = 0.1737 + 0.0996x \quad \text{(I)}$$

$$y = 0.1740 + 0.0936x \quad \text{(II)}$$

$$y = 0.1816 + 0.1026x \quad \text{(III)}$$

where $y$ is the absorbance of the suspension at 540nm and $x$ (g/L) is the biomass concentration.

The $R^2$ of the three equations that represented batch 1, 2 and 3 were 0.948, 0.941 and 0.941, respectively. The linear fit can be used to represent the relationship of OD and cell concentration in either old strains batch or new strains batch in this study and it is reasonable enough to explain an approximate tendency of cell concentration during an algae growth batch cycle. This relationship can help with predicting experimental results.

### 3.3.4 Effects of different strains of *C. protothecoides* and different substrates on the results of accumulated biomass and lipid productivity

Comparing the BM-GY substrate and mixed waste substrate experiments, the results related to biomass and lipid productivities were not similar. As shown in figure 3.7 and table 3.3, in the BM-GY group, the accumulated biomass productivities of the old strains batch and new strains batch were 2.07 (g/L/day) and 1.61 (g/L/day) on the last day, respectively. And the accumulated biomass productivities of the mixed waste group achieved 2.12 (g/L/day) in the old strains batch and 1.81 (g/L/day) in the new strains batch. The accumulated biomass productivities of the old strains did not perform significant difference between the BM-GY group and the mixed waste group (t-test, p-value=0.3712), however, the new strains used in this study appeared significant higher biomass productivities in the mixed waste group than those in the BM-GY group (t-test, p-value=0.0271). Comparing the overall biomass productivities of old strains and new strains, the results indicate that in both of the two different substrates, the old strains accumulated higher biomass productivities than the new strains.

As summarized in Table 3.3, the accumulated lipid productivities on the seventh day of the old strains batch yielded 0.91 (g/L/day) in the BM-GY group and 0.94 (g/L/day) in the mixed waste group. The lipid productivities of the new strains in the two substrates were 0.82 (g/L/day) and 0.95 (g/L/day), separately. From the figure 3.7, the old strains achieved similar accumulated lipid productivities in the BM-GY and waste cultures (t-test, p-value=0.1110), while the new strains could reach higher lipid productivities in the mixed waste

medium (t-test, p-value=0.0050). There are no significant differences of lipid productivities between the old and new strains in the waste group. However, in the BM-GY group, the accumulated lipid productivities of the old strains were higher than those of the new strains.

Based on the data analysis of different substrates and different strains, it is said that the accumulated biomass and lipid productivities of the old strains presented similar results in BM-GY and mixed waste substrates. The new strains can achieve higher biomass and lipid productivities in the mixed waste group. By contrast of the biomass and lipid productivities, the different strains were considered as a reasonable factor that might explain the dissimilar variation trends of their productivities. This can be demonstrated by the photomicrographs (Figure 3.6). Figure 3.8 indicated that the old strain showed a smaller average cell size at log phase compared with new strain cell size, but the concentration of old cells under microscope were much higher than that of the new strain. Since the old and new strains were *Chlorella protothecoides* UTEX 256 obtained from exactly the same source (the culture collection of algae at University of Texas), one assumption of the results was possible mutations or changes within the old strain that created some changes of species characteristics. Mutation is identified as DNA sequences changing by outside conditions of an organism and random mutations happen with the evolution of biology. Many possible conditions can induce a mutation in an organism such as ultra-violet light (Srivastava 1969), and chemical agents (Costas, et al. 2013). Some studies attempt to apply mutagenesis technology into biofuel production to reduce the cost and increase the production (Ma, et al. 2013).

## Tables and Figures

Table 3.1 Elemental composition of brewer fermentation waste by ICP analysis

| Elements | Stock I[a] Parts per million (ppm) | Stock II (ppm) | Stock III (ppm) |
|---|---|---|---|
| Aluminum | 0.453 | 0.311 | 3.976 |
| Boron | 0.428 | N/A | 0.915 |
| Calcium | 28.32 | 35.53 | 180.25 |
| Copper | 0.063 | 0.168 | 2.482 |
| Iron | 0.634 | 0.922 | 10.476 |
| Potassium | 942.3 | 838.4 | 1253.6 |
| Magnesium | 138.9 | 130.3 | 215.0 |
| Manganese | 0.82 | 0.52 | 1.56 |
| Sodium | 31.39 | 20.63 | 29.02 |
| Phosphorus | 575.6 | 426.1 | 779.0 |
| Sulfur | 153.2 | 160.3 | 350.7 |
| Zinc | 1.932 | 0.143 | 3.205 |
| TKN(%) | 0.264 | 0.145 | 0.527 |

[a] Used as the experimental fermentation waste stock in this research

Table 3.2 Elemental composition of biodiesel by-product crude glycerol by ICP analysis

| Elements | Stock I[a] Parts per million (ppm) | Stock II (ppm) | Stock III (ppm) |
|---|---|---|---|
| Aluminum | <3.000 | <3.000 | <3.000 |
| Boron | N/A | N/A | N/A |
| Calcium | 13.06 | 30.33 | 17.48 |
| Copper | 1.953 | 6.311 | 1.505 |
| Iron | 8.753 | 10.648 | 8.043 |
| Potassium | 3535.87 | 36236.1 | 31375.5 |
| Magnesium | 3.262 | 17.963 | 0.689 |
| Manganese | 0.158 | 0.242 | 0.212 |
| Sodium | 120.514 | 271.519 | 382.223 |
| Phosphorus | 10.531 | 79.129 | 188.309 |
| Sulfur | 3564.62 | 20.61 | 28.30 |
| Zinc | 4.051 | 15.784 | 0.478 |
| TOC(%) | 30.55 | 22.64 | N/A |

[a] Used as the experimental crude glycerol stock in this research

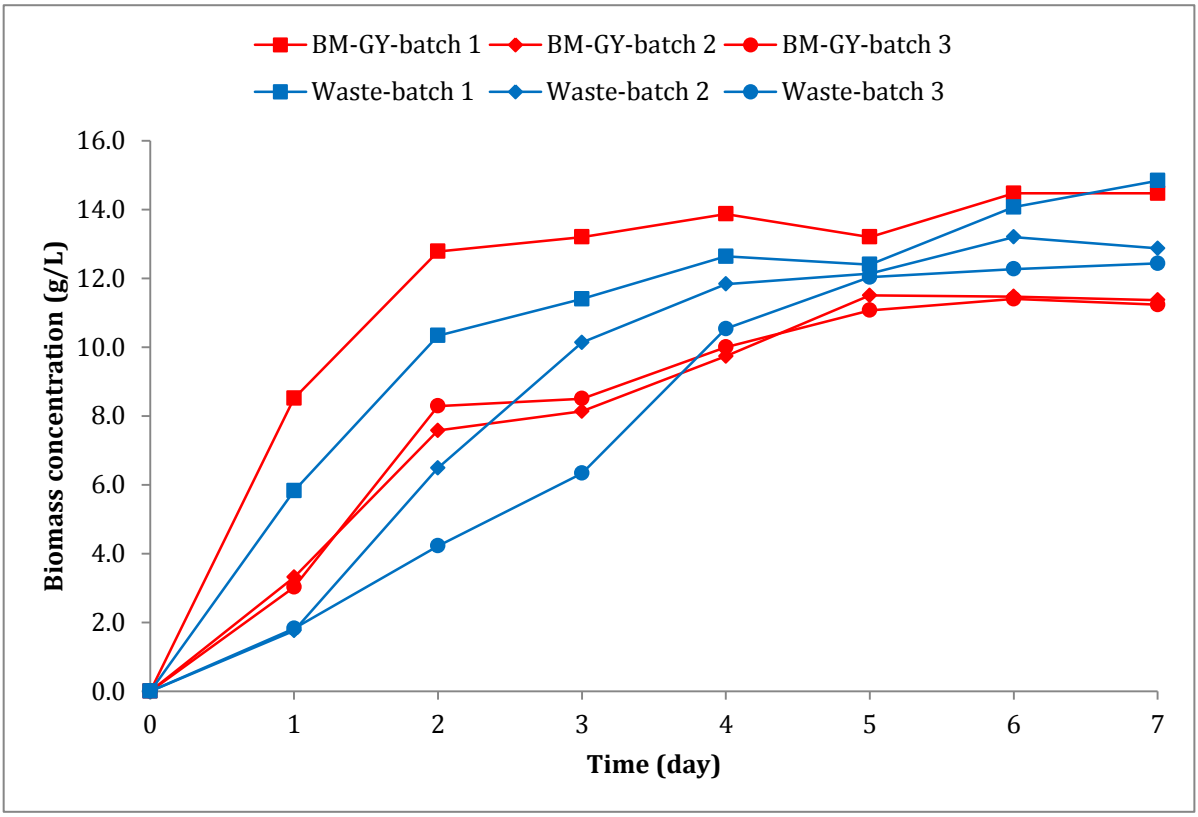



Figure 3.1 Biomass accumulations of *Chlorella protothecoides* within three batches of BM-GY group and mixed waste group

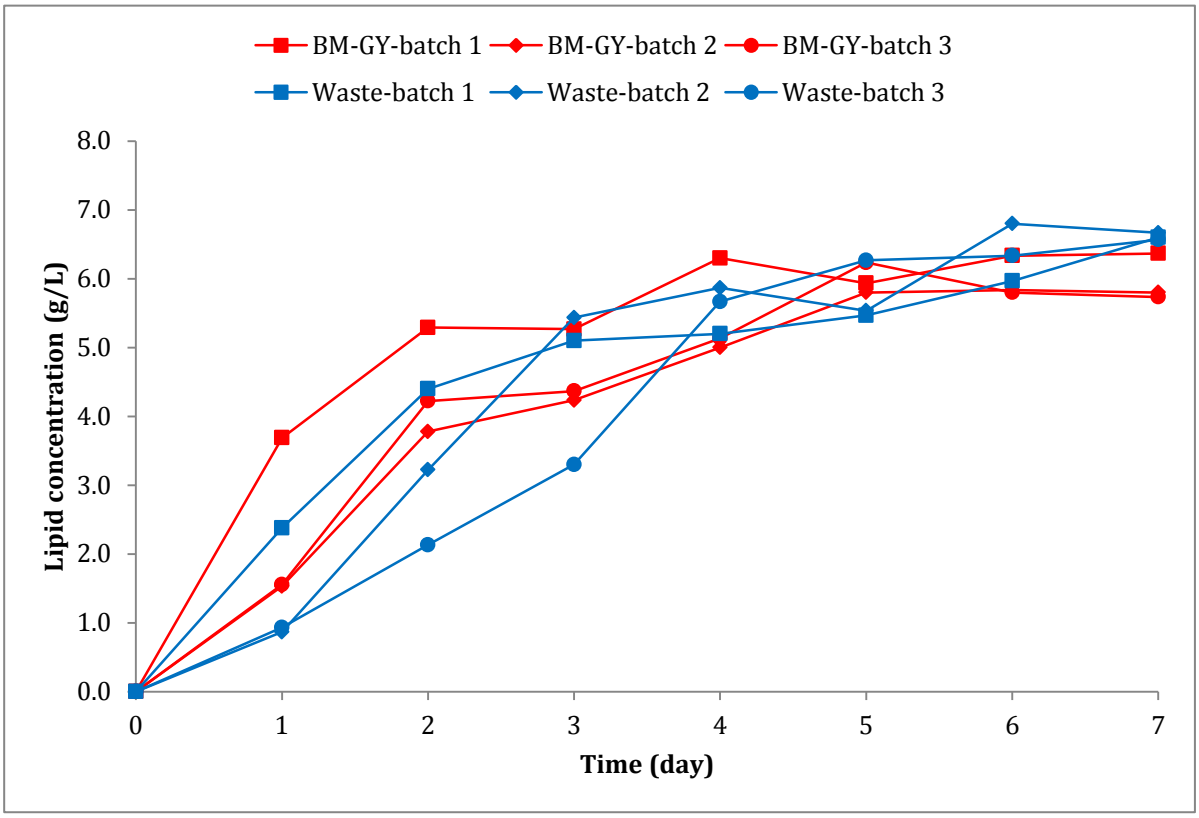



Figure 3.2 Lipid accumulations of *Chlorella prototheco*ides within three batches of BM-GY group and mixed waste group

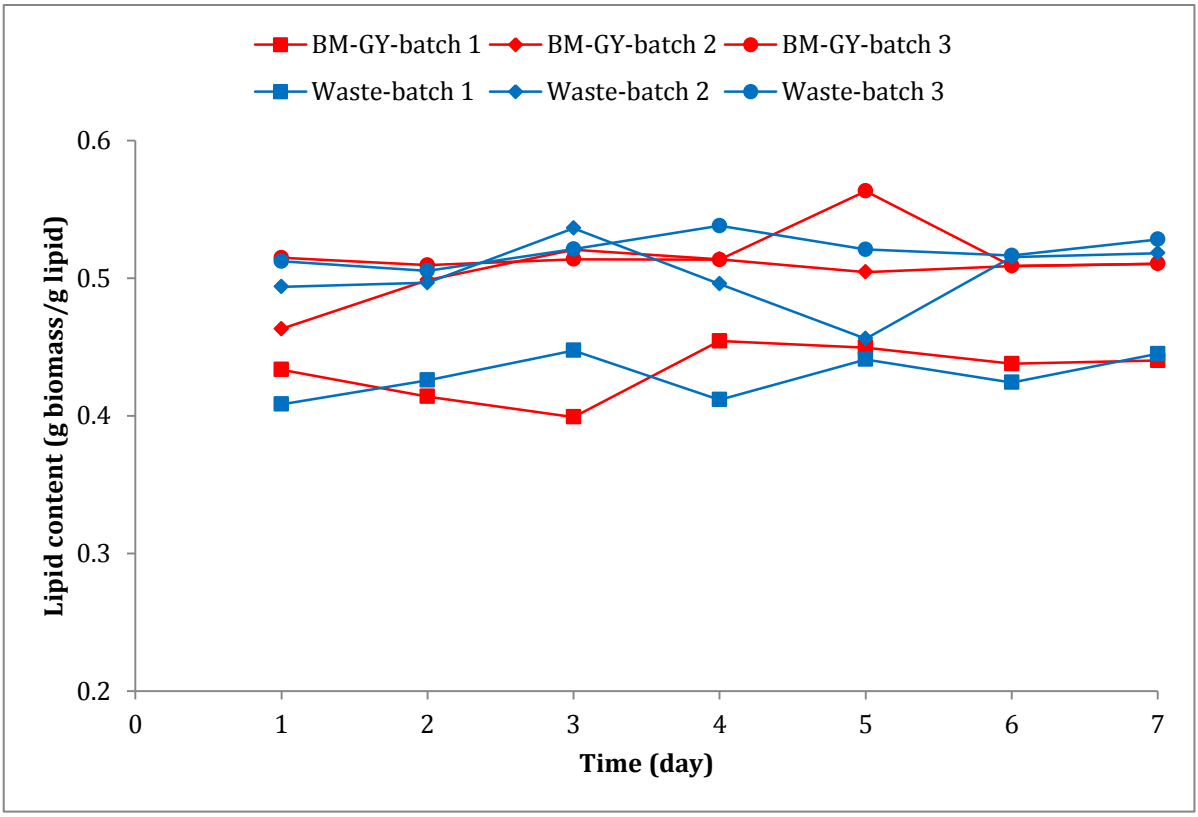
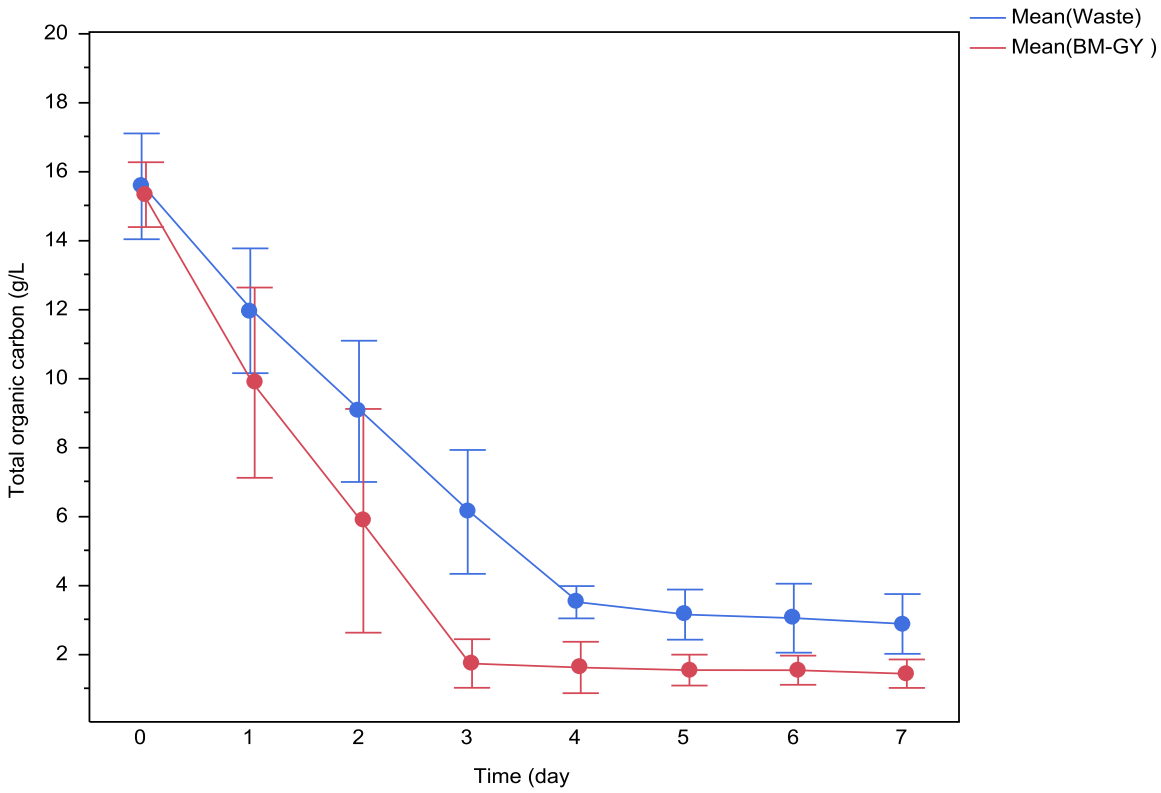


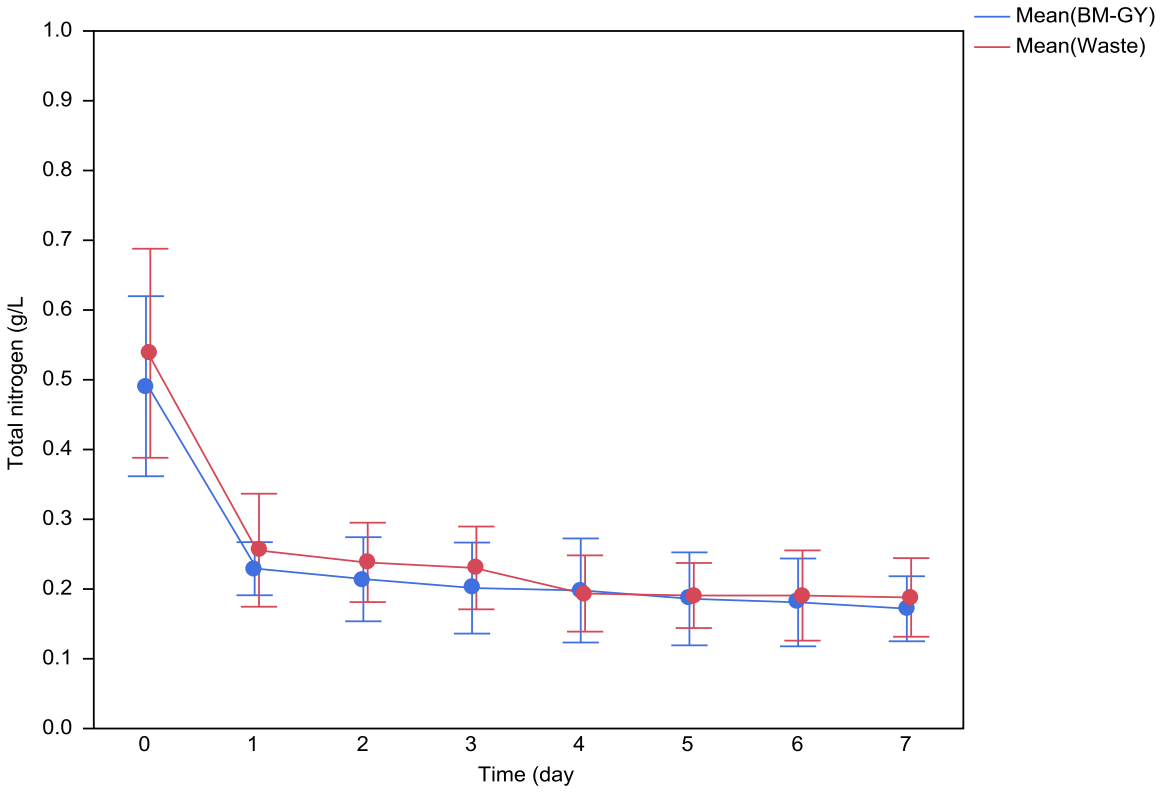

Figure 3.3 Lipid contents of *Chlorella protothecoides* within three batches of BM-GY group and mixed waste group




Each error bar is constructed using 1 standard deviation from the mean.

Figure 3.4 Consumption of total organic carbon (TOC) of *Chlorella protothecoides* in BM-GY group and mixed waste group

Each point is the mean value of triplicate measurements of three parallel batch experiments




Each error bar is constructed using 1 standard deviation from the mean.

Figure 3.5 Consumption of total nitrogen (TN) of *Chlorella protothecoides* in BM-GY group and mixed waste group

Each point is the mean value of triplicate measurements of three parallel batch experiments

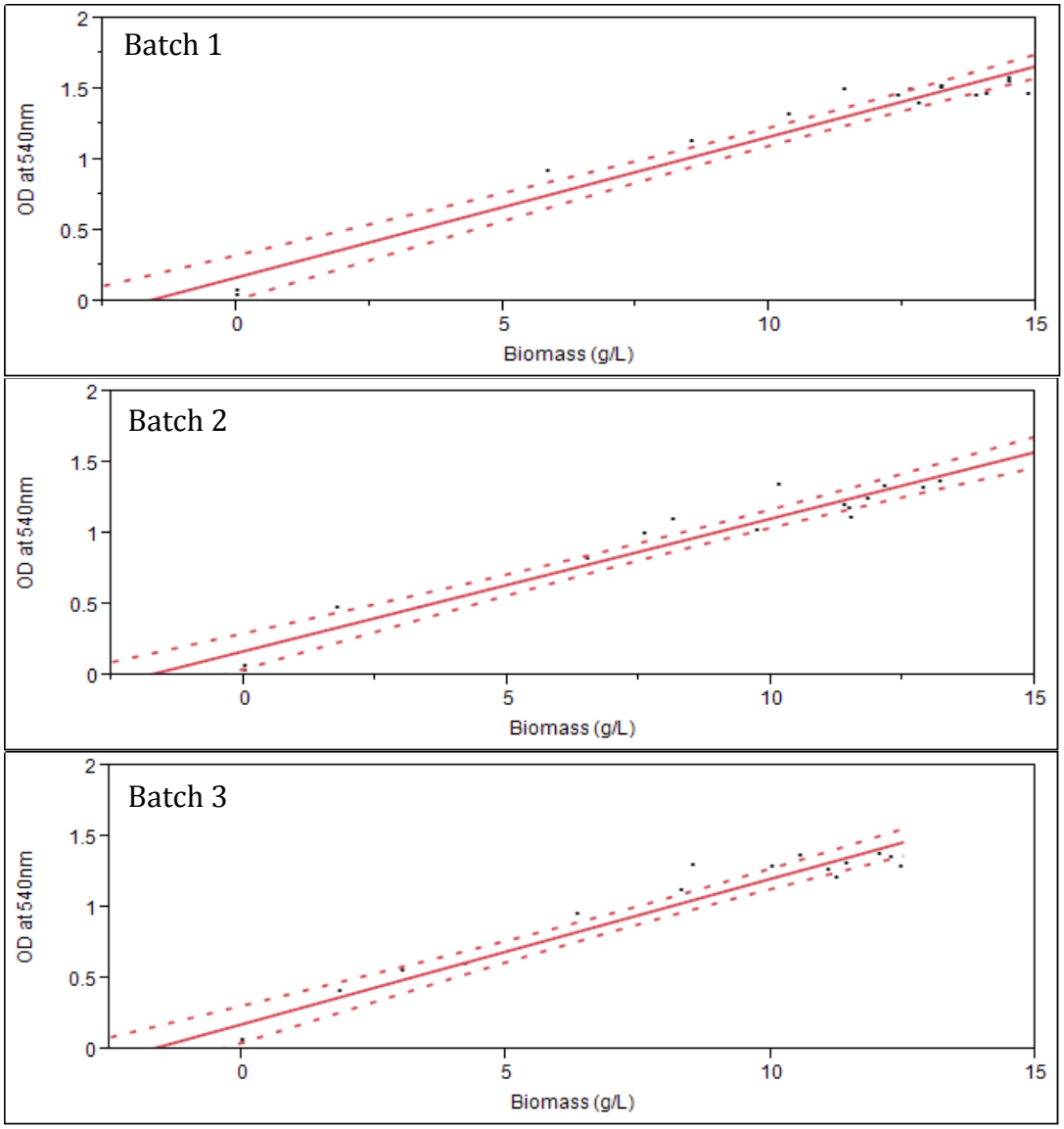
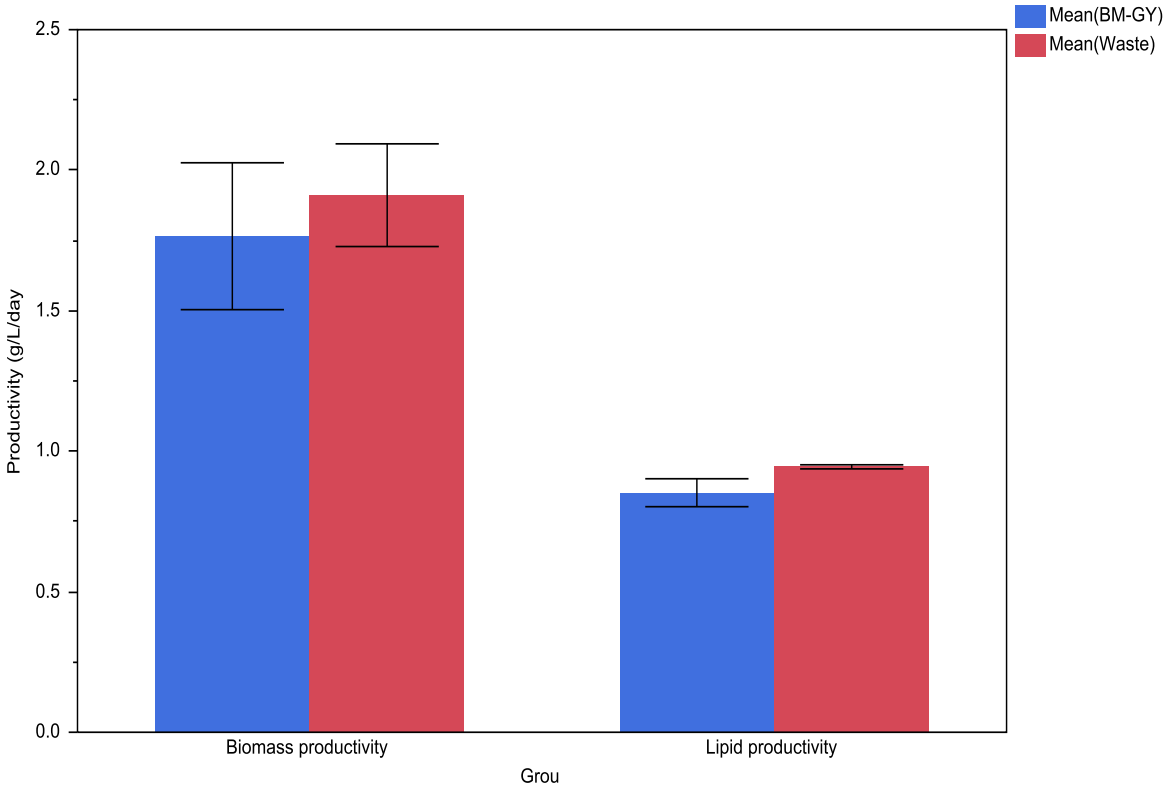



Figure 3.6 Optical density and biomass concentration correlative linear fit model test of three batches




Each error bar is constructed using 1 standard deviation from the mean.

Figure 3.7 Accumulated biomass productivity and lipid productivity of *Chlorella protothecoides* in BM-GY group and mixed waste group on seventh day

Each bar is the mean value of triplicate measurements of three parallel experiments

Table 3.3 Comparison and summary of accumulated biomass and lipid concentration and productivity of old and new Chlorella protothecoides strains in BM-GY medium and mixed waste medium

| Substrates | Max biomass concentration (g/L) | Accumulated biomass productivity (g/L/day)[c] | Max lipid concentration (g/L) | Accumulated lipid productivity (g/L/day)[c] |
|---|---|---|---|---|
| **BM-GY for old strains[a]** | 14.46 | 2.07 | 6.37 | 0.91 |
| **Mixed Waste for old strains[a]** | 14.83 | 2.12 | 6.60 | 0.94 |
| **BM-GY for new strains[b]** | 11.43±0.047 | 1.61±0.013 | 6.02±0.306 | 0.82±0.007 |
| **Mixed Waste for new strains[b]** | 12.73±0.660 | 1.81±0.044 | 6.62±0.071 | 0.95±0.010 |

[a] Data shown are the mean values of triplicate samples of batch 1 grown with old C. protothecoides strains
[b] Data shown are the mean values of triplicate of batch 2 and batch 3 grown with new C. protothecoides strains ± standard deviations
[c] Cell growth data from seventh day were used for calculation of accumulated productivities.

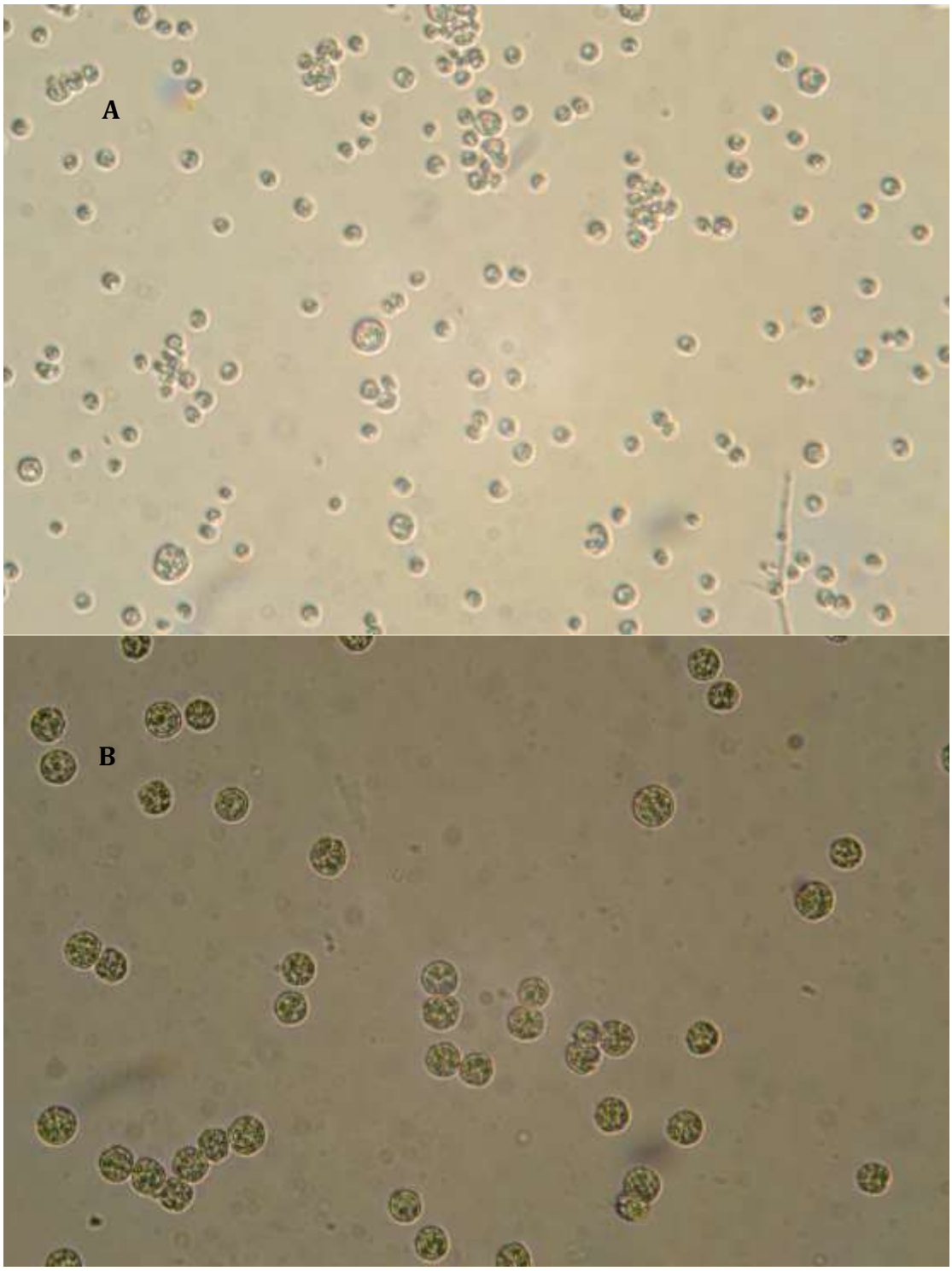



Figure 3.8 Photomicrographs of old strain (A) and new strain (B) *Chlorella protothecoides* cells grew in waste medium at the middle of the process

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# CHAPTER IV

# CONCLUSION AND IDEAS FOR FURTHER RESEARCH

## 4.1 Conclusion

The results of this study demonstrate that (1) *Chlorella protothecoides* can utilize the carbon from biodiesel crude glycerol as a carbon substrate and use the nitrogen from brewer fermentation waste as a nitrogen substrate. Other required trace elements for algae growth can also be obtained from the mixed waste to support cell growth; (2) The new strains grew better and accumulated more biomass in the mixed waste substrate under heterotrophic batch mode fermentation. However, the old strains always reached higher biomass concentrations than the new strains in both of the two different substrates. Meanwhile, the total organic carbon and total nitrogen in the waste streams can be removed by algae; (3) The new strains can achieve higher accumulated lipid productivity in the mixed waste group and the old strains have the similar lipid productivity in the BM-GY and the mixed waste groups. This result indicates that the brewer fermentation waste and biodiesel crude glycerol are valuable alternative substrates for biofuel production and may lower the capital cost in biodiesel industry and achieve the similar or higher lipid productivity; (4) Optical density (OD) can be regarded as a reasonable indicator to reflect the biomass accumulation tendency and the functional equation tested in this study was a linear fit; (5) An old *C. protothecoides* strain kept for quite a long time may appear some characteristic changes caused by destabilizing factors and

uncertainties in the external environment that may alter and influence the biomass and lipid accumulation and the productivities when compared to the results of a new *C. protothecoides* strain. This assumption may need further study on the gene level to provide more information.

### 4.2 Further research

Based on the studies in this experiment, the further research may focus on (1) how to select and control the waste streams used for algae growth since the waste streams always have large variations on their elements; (2) other reasonable cheap waste streams that can be easily obtained to use as algae substrates; (3) large scale application of using waste streams as substrates for biofuel and wastewater treatment; (4) the impact of mutated strains on algae growth and the possibility of applying the mutations to enhance the biomass concentration and lipid productivity.